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[54] 发明名称 可用于大规模生产的重组腺病毒伴随病毒生产方法及用途

[57] 摘要

本发明提出一种“一株载体细胞/一株辅助病毒”生产重组 AAV 病毒的方法。其中载体细胞株是将含有外源基因表达单位的重组 AAV 载体质粒导入哺乳动物细胞，经抗性筛选获得的整合了可拯救的重组 AAV DNA 的稳定传代的细胞株。辅助病毒是携带 AAV rep/cap 基因的重组 HSV－1 病毒。用这种全功能辅助病毒感染载体细胞株即可产生大量有感染性的重组 AAV 病毒。本发明可用于重组 AAV 病毒的规模化高效生产。

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Method for large-scale production of recombinant adeno-associated virus and uses thereof

The present invention belongs to the field of biotechnology, and relates to the method for producing recombinant adeno-associated virus and the use of the product obtained thereform, particularly to the use in the field of gene therapy and transgenic animals. The invention is a continuation of the Chinese patent application Nos. 98101753.3 (application number) and 98120033.8 (application number).

Gene delivery system is the key technology of gene therapy research and application, including viral vector system and non-viral vector system. Viral vectors mainly include retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, herpes simplex virus vectors and the like. Among others, Adeno-associated virus (AAV) vector is highly regarded for its advantages of security, stability, infecting both dividing cells and non-dividing cells , highly infecting efficiency, long-term expression of exogenous gene and so on.

AAV is a type of non-pathogenic tiny virus that has a genome of 4680nt singal-stranded DNA. The life cycle of AAV has two types of dormant infection and lytic infection. The lytic replication of AAV needs the participation of helper virus such as adenovirus or herpes simplex virus (HSV). AAV will integrate into the chromosome of the host cell in the form of provirus in the absence of helper virus. At both ends of the chromosome of AAV exists 145nt ITR sequence respectively, which is the necessary cis-acting element of AAV and plays an important role in such functions as the rescue, replication, package and integration of the AAV genome, etc. Between two ITRs are trans-acting genes rep and cap, which are essential for the replication of AAV DNA and the production of the virus particles. Rep gene encodes four types of proteins: Rep78, Rep68, Rep52, and Rep40, which are associated with such functions as replication, integration, rescue of AAV, etc. Cap gene encodes three types of capsid proteins, VP1, VP2, and VP3, which constitute the icosahedron capsid of AAV.

Samulski RJ *et al* (Cloning of adeno-associated virus into pBR322 : rescue of intact virus from the recombinant plasmid in human cells, Proc. Natl. Sci. USA, 79: 2077-2031,1982) found that the genome of AVV provirus in plasmid pBR322 still has infectivity upon cloning the full-length double-stranded DNA of AAV into the plasmid. A recombinant adeno-associated virus (rAAV) that contains exogenous gene thus can be obtained in cells when the expression unit of the exogenous gene is located between two ITRs of AAV, and the functions of rep and cap genes as well as helper virus are provided in other trans-acting ways.

The classical method for rAAV production is co-transfecting 293 cells with double plasmids and infecting the cells with helper virus such as type 5 adenovirus (Ad5). One of the double plasmids is recombinant AAV vector plasmid, and the other is helper plasmid that contains rep/cap gene of AAV. Many researchers dedicate to improve the production method of rAAV due to the complexed nature of the operation, the various factors affecting the production of rAAV, as well as the difficulties in obtaining high titer rAAV and in large- scale production. These improvements include the following classes: 1) rep/cap gene is transduced into a cell strain to establish a packaging cell line, wherein the expression of rep/cap gene is controlled by the promoter of itself or by other constitutively or inducible promoters.

2) AAV ITRs, together with the expression unit of the exogenous gene DNA between the ITRs, are inserted into the genome of adenovirus to construct a chimeric recombinant adenovirus. 3) AAV ITRs, together with and the expression unit of the exogenous gene DNA between the ITRs, are located in an autonomously replicating EBV vector, and then transduced into cells to generate a cell strain which carry a recombinant AAV vector plasmid that can replicates autonomously. 4) rep/cap gene is inserted into the genome of adenovirus to construct a recombinant adenovirus which has entire helper function. However many experiments have demonstrated that this approach can hardly come true possibly because the strongly inhibiting function of rep protein to adenovirus make the recombinant adenovirus that contains rep/cap gene unable to be produced. 5) The amplicon of HSV is used to carry rep/cap gene to produce HSV mixed virus that has entire helper function.

Although every improvement on the production of rAAV have increased the yield of rAAV in various degree or simplified the production method of it, these methods have not yet well solve the problem of producing rAAV on a large scale with high efficiency. The cost (including time cost and money cost) of producing rAAV that is enough for human experimental amount is still very high by now. Therefore, it is very necessary for inventing a method that can be used to produce rAAV on a large scale and efficiently.

The purpose of the invention is to provide a method that can be used to produce recombinant adeno-associated virus (rAAV) vector on a large scale, that is "a method of producing "one vector cell / one helper virus". The method has the characteristics of high production yield, simple operation, and is easy for scale-up. The invention can be used to produce recombinant AAV in large amount, and the recombinant AAV produced therefrom can be used in gene therapy for various diseases.

A traditional method of producing rAAV virus is co-transfecting 293 cells with double plasmids and infecting the cells with helper virus (type 5 adenovirus). The double plasmids include a recombinant AAV vector such as pAB11, and a helper vector that contains rep/cap gene of AAV such as pAd8. This method has the disadvantages of low transfection efficiency, instability, complexed operation, or difficult to enlarge scale and process due to the transfection of cells. Although this method has been improved, such as the transfection efficiency of 293 cells can reach or approach 100% by using the method of calcium phosphate coprecipitation, yet it is difficult for this method to be used in a large amount of cells, and the request on 293 cells themselves (generation is less than 50, cell is flat, and doubling time is 36hr-48hr) restrict the obtainment of bulk of rAAV by this method.

Aiming at the disadvantages of many influencing factors, complex operation, and unable to be produced on a large scale during the production process of rAAV, accordingly difficult to obtain high yield of rAAV presently , the invention provides a production strategy of "one vector cell/one helper virus": The production cell strain that integrates the DNA of the recombinant AAV vector stably is infected by using the recombinant HSV-1 (HSV1-rc) which carries the rep/cap gene of rAAV to obtain a large amount of rAAV from pathologically changed cells. HSV1-rc may provide simultaneously the helper virus HSV-1 that are necessary for the packing of rAAV, as well as the trans-acting protein Rep

and the capsid protein, which two functions are usually provided by wild-type helper virus such as Ad5 or HSV-1, and helper vector that contains rep/cap gene such as pAds. The recombinant genome DNA (single-stranded) that is packaged into the particles of the recombinant AAV is from the recombinant AAV vector cell strain. One or more copies of the recombinant AAV vector DNA are integrated into the genome of the recombinant AAV vector cell stably, in which two ITR sequences of AAV and the expression unit of therapeutic gene between the two sequences are comprised. The expression unit of therapeutic gene consists of eukaryotic expression promoter (such as HCMV IE promoter, SV40 promoter, β-globin promoter, inducible promoter, various tissue specific promoters and the like), therapeutic gene, mRNA tailing signal, with the length less than 5.0kb.

When using rHSV-rc to infect vector cell strain, The DNA of HSV1-rc replicates in large amount and produces progeny virus finally upon entry into the cells. When the DNA of HSV1-rc replicates, the rep/cap gene in it replicates simultaneously to produce high copies of rep/cap gene. The rep gene encodes 4 types of Rep proteins (Rep78, Rep68, Rep52, Rep40), by which the DNA of the recombinant AAV vector is rescued out of the cell genome and replicates largely and packaged into AAV particles finally as a single strand. The cap gene encodes 3 types of capsid proteins VP1, VP2, VP3, which are assembled to be a particle in cell nucleus.

The process of producing rAAV of the invention is similar very much with the process of lytic infection and replication of the wild-type AAV in the case of HSV-1 as the helper virus. The difference between the two processes is that between the ITRs of the genome of the wild-type AAV is rep/cap gene and the helper virus is wild type HSV-1; whereas in the invention, rep/cap gene is inserted into the genome of the helper virus HSV-1, resulting in a recombinant HSV-1 virus rHSV1-rc that has the entire helper function, and the ITRs of AAV and the therapeutic gene between ITRs are provided by vector cell strain. Infecting a productively monoclonal cell strain of rAAV (screened from the vector cell clones) with rHSCV1-rc, may obtain the similar production efficiency of rAAV with the wild-type AAV that is helped by the wild-type HSV-1 under certain condition. The production efficiency of rAAV may reach 10^{4-5} particles rAAV/cell when using BHK cell as the production cell.

There are four steps for the high production of rAAV: a) The high production of HSV1-rc. It is easy for the virus to be cultured largely on BHK cells or other sensitive cells due to the ability of replication thereof, the infected cells will have obvious pathological changes and there exists a lot of infectious HSV1-rc both in the supernatant and in the cells. The titer of HSV1-rc in the supernatant may reach 10^{7}pfu/ml., and 10^{8}pfu/ml HSV1-rc may be obtained by the method of freeze-thaw to disrupt cells. Upon low speed centrifugation to remove cell debris, the acquired virus supernatant could be used for the next step; b) The large culture of the vector cell strain. The equipments and methods that may be used in the culture are spinner culture, cell fermentor culture as well as other high density cell culture; c) Infection the vector cell strain above with HSV1-rc. There are a lot of rAAV in the cell lysate after the completely pathological changes of the cells. d) The isolation and purification of rAAV.

The invention relates to the production of the recombinant HSV1-rc, HSV-1 that has the rep/cap gene of AAV virus, and to the establishment of the production cell strain that has the recombinant AAV vector. The production method of HSV1-rc

and the use thereof have been described in Chinese patent application 98120033.8 (application number). The production of HSV1-rc is carried out based on the genetic operation of a set of cosmid (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) that has been inserted of 5 fragments from the whole genome of HSV1 (there are partial overlaps at the terminal sequence sequentially). Firstly, the rep/cap gene of AAV-2 is inserted into the genome of HSV-1 in one of the cosmids by recombinant DNA technology, for example to be inserted into the HSV1 UL2 gene in cos6 to construct cos6-rcΔUL2; or to be inserted into the HSV1 UL44 gene of cos56 to construct cos56-rcΔUL44. It is also feasible to insert rep/cap gene into other sites of the genome of HSV-1. The processes mentioned above are carried out in E. coli. The recombinant cosmid DNA into which the rep/cap gene is inserted and the rest of four corresponding cosmids DNA are cut together with Pac□enzyme (cutting the backbone part of the cosmids). The cut DNA is extracted and purified with phenol/chloroform, then is transfected into BHK-21 cell or other sensitive cells to HSV1 by the method of lipofectamine or other transfection methods. The pathological changes and plaques may be observed after 5 to 7 days, it shows that homologous recombination of the five fragments occurs in the cells and the recombinant HSV1 is produced. Single plaque is detected individually, including the detection by PCR method for rep gene and cap gene fragments, and the function for rAAV package . The positive result shows the recombinant HSV1 (HSV1-rc) into which the rep/cap gene is inserted. The plaques of the obtained HSV1-rc are purified two times to ensure their purity.

The construction of the production cell strain, namely the vector cell strain, which has the recombinant AAV vector: The plasmid which has the recombinant AAV vector DNA (AAV ITRs and the expression unit of therapeutic genes between them) is introduced into cells by lipofectamine or other transfection methods , and resistant cell clones are obtained by selective culture with neomycin analogs G418. The monoclonal cells are selected individually and amplified. The stably continuous cell strains that can produce high titer rAAV under the infection of HSV1-rc are screened, froze and stored largely until use. The selective gene neo may be located on the recombinant AAV plasmid vector, between the AAV ITRs, or in the plasmid backbone outside of ITRs, and neo may also be located in the other plasmid such as pSV2neo, and be co-transfected into cells with recombinant vector plasmid.

BHK-21 cell is a nice rAAV production cell strain in the method of the invention. Other cell strains such as 293 cell, KB cell, HeLa cell may also be used for producing rAAV.

The rAAV that is produced in the invention may carry various target genes and may be used in gene therapy for genetic diseases, tumors, cardiovascular diseases, and infectious diseases.

The advantages of the invention:

The methods provided in the invention may be used for the large-scale production of rAAV. The strategy of "one vector cell strain/one helper virus" used in the invention overcomes the disadvantages of complex operation, variou affecting factors or difficulties in scale up of traditional methods and modifications thereof, and simplifies greatly the affecting factors for rAAV production and the production process thereof, wherein both the vector cell strain and the full function helper virus HSV1-

rc have the characteristics of stable passage and amplification, which are benefit for enlarging the production. The rep/cap gene of AAV may replicate with the replication of the helper virus HSV1-rc to become high copies during the production process of rAAV, which simulates the replication process of wild-type AAV, and is benefit for the production of high titer rAAV.

The microbial strains related to in the invention

The microbial strains that relates to in the invention during the production process of HSV1-rc include DH5α/cos6-rcΔUL2 and DH5α/cos56-rcΔUL44 which are deposited in China General Mcirobiological Culture Collection Center on September 24, 1998. The accession numbers are CGMCC No.0361-1 and CGMCC No.0361-2, respectively.

Examples: The method that can be used for producing recombinant adeno-associated virus on a large scale and the use thereof are specified in the following examples, which should not be construed as limitations for the contents of the invention.

Example 1 The construction of the recombinant AAV vector plasmid which carries reporter gene GFP

1) The construction of pSNAV-1/GFP

The gene GFP (green fluorescence protein) is derived from pGreen Lantern-1 (GIBCO BRL, #10642-015). The universal AAV vector plasmid pSNAV-1 is constructed in our laboratory (Chinese paten application number: 99119038.6).

The plasmid DNA pGreen Lantern-1 was cut by NotI, the fragment GFP was extracted and inserted into the corresponding site in the plasmid pcDNA2.1 to construct pcDNA2.0/(GFP(+/-). The recombinant plasmid which had the opposite orientation between GFP transcription and T7 promoter was named pcDNA2.0/GFP(-). The pcDNA2.0/GFP(-) was double digested by EcoR I and Sal I, the fragment GFP was extracted and inserted into the site between EcoR □ and Sal □ in pSNAV-1 to construct pSNAV–1/GFP. The plasmid comprised in turn the following elements:

ITR-CMV-GFP-SV40 polyA-ITR-SV40 promoter-neo-polyA-amp^R-E.coli ori.

2) The construction of pSNAV-2/GFP

The gene GFP (green fluorescence protein) is derived from pGreen Lantern-1 (GIBCO BRL, #10642-015). The universal AAV vector plasmid pSNAV-1 is constructed in our laboratory (Chinese paten application number: 99119038.6).

The plasmid DNA PcDNA2.1A/GFP(-) was double cut by Kpnland Xhol, the fragment GFP was extracted and inserted into the site between Kpn□ and Sal □ in pSNAV-2 to construct pSNAV–2/GFP. The plasmid comprised in turn the following elements:

ITR-CMV-GFP-SV40 polyA-SV40 promoter-neo-polyA-ITR-amp^R-E.coli ori.

The difference between pSNAV–2/GFP and pSNAV–1/GFP is that the expression unit of neo gene is located between two ITR sequences in

pSNAV–2/GFP, whereas the expression unit of neo gene is located out of the two ITR sequences in pSNAV–1/GFP.

Example 2 the construction of AAV-GFP vector cell strian

The plasmids pSNAV–1/GFP and pSNAV–2/GFP were used individually to transfect BHK cells, and two AAV-GFP vector cell strains BHK/pSNAV-1/GFP and BHK/pSNAV-2/GFP were obtained after selective culture.

BKH cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C. The plasmids BHK/pSNAV-1/GFP and BHK/pSNAV-2/GFP were used individually to transfect BHK cells with lipofectamine (GIBCO BRL), digested after 24 hours and subcultured at 1:2-5. Resistant cell clones appeared clearly after 10 days with 800μg/ml G418 selection. The cell clones were observed with inverse fluorescent microscopy, and 20 single cell clones with GFP gene expression were selected each for scale up and freeze preservation. Each cell clone was infected with the full function helper virus HSV1-rc, and the detection results showed that recombinant AAV-GFP could be produced in various degree by each cell strain. The titer of the recombinant AAV-GFP was detected respectively, and the cell strain that had a high titer of recombinant AAV was selected as AAV-GFP vector cell strain. The genome DNA (single strand) in the recombinant AAV virus particle that was packaged by the vector cell strain BHK/pSNAV-1/GFP was about 1600nt, and consist of two ITRs and CMV-GFP-SV40 between them. Whereas The genome DNA (single strand) in the recombinant AAV virus particle that was packaged by vector cell strain BHK/pSNAV-2/GFP was about 3700nt, and consist of two ITRs and CMV-intron-GFP-SV40 polyA-SV40 promoter-neo-polyA between them.

Example 3 the production of the recombinant AAV-GFP

Cells were cultured in RPM1640 medium supplemented with 10% fetal bovine serum at 37°C. 5×10^6 vector cells BHK/pSNAV-1/GFP were inoculated in 15-cm culture plate, and cultured in an incubator at 37□ , 5% CO_2 for 48 hours. 1ml (moi=0.5-1) full function helper viruses HSV1-rc were added in the plate to absorb for 1 hours. RPMI1640 medium supplemented with 2% fetal bovine serum was added to 30ml/plate and cultured at 37□ for 36-48 hours. The supernatant and the cells were collected, and the cells were disrupted for four times by freezing and thawing cycles and centrifuged at 1500rpm for 10min, the supernatant was collected and the helper virus was inactivated at 56°C. The rAAV-GFP were concentrated with ammonium sulfate and purified by $CrCl_2$ gradient centrifugation , and stored at 4□ or –70°C by adding 5% glycerin or sucrose after dialyzing in PBS buffer for desalting.

What we claimed is:

The present invention relates to the field of biotechnology, particularly to the production and use of the recombinant adeno-associated virus

vector (rAAV) for the use in gene therapy.

1. The method of the present invention is characterized by the utilization of the strategy of "one cell/one virus" for production of rAAV virus.

2. A method of using "one vector cell/one full function helper virus" to produce rAAV virus, which comprises:

(a) establishing the recombinant AAV vector cell strain;

(b) generating and preparing the full function helper virus;

(c) infecting the vector cell strain with the full function helper virus to produce rAAV virus.

3. (The method of claim 2, wherein) the stably continuous cell strain of the recombinant AAVis obtained by introducing the recombinant AAV vector plasmid into a mammalian cell, and culturing them selectively.

4. (The method of claim 2, wherein) the AAV ITRs and the expression unit of therapeutic gene are integrated stabely in the chromosome of the vector cell strain.

5. (The method of claim 2, wherein) the rep/cap gene of AAV is inserted into the genome of herpes simplex virus to construct a full function helper virus.

6. (The method of claim 2, wherein) the rAAV produced carries target gene useful in gene therapy for genetic diseases, tumors, cardiovascular diseases, and infectious diseases.

7. The method according to claim 4, wherein the vector cell is derived from BHK cell.

8. The method according to claim 4, wherein the vector cell is derived from KB cell, 293 cell, and HeLa cell.

9. The method according to claim 4, wherein the antibiotic resistance gene is provided by recombinant AAV vector plasmid.

10. The method according to claim 4, wherein the resistance gene is provided by another plasmid.

11. The method according to claim 4, wherein the resistance gene is neo.

12. The method according to claim 4, wherein the resistance gene is hph.

13. The method according to claim 6, wherein the rep/cap gene of AAV is inserted into the genome of type I herpes simplex virus (HSV-1).

14. The method according to claim 6, wherein the rep/cap gene of AAV is inserted into the genome of type□herpes simplex virus (HSV-1).

15. The method according to claim 6, wherein the rep/cap gene of AAV is inserted into UL2 and/or UL4 in the genome of type I herpes simplex virus (HSV-1).

16. The method according to claim 6, wherein the rep/cap gene of AAV is inserted into any site in the genome of herpes simplex virus (HSV-1).

ABSTRACT

The present invention presents a strategy of "one vector cell /one helper virus" to produce recombinant AAV. The vector cell strain is a stably continuous cell strain that integrate a rescuable recombinant AAV DNA, which is obtained by introduction of the recombinant AAV vector plasmid containing the expression unit of the exogenous gene into mammalian cell followed by resistant screen. The helper virus is a recombinant HSV-1 that carries the rep/cap gene of AAV. A large amount of recombinant AAV that are infectious can be produced by infecting the vector cell strain with the full function helper virus. The present invention can be used for the production of the recombinant AAV on a large scale.